DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

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and

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PREPARATION OF PHA-STIMULATED UNINFECTED DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS

I. PRINCIPLE

Peripheral blood mononuclear cells (PBMC) are isolated from healthy, uninfected donor blood for use in various assays and to culture HIV. The PBMC are stimulated with the mitogen phytohemagglutinin-P (PHA-P), in the presence of human interleukin 2 (IL-2) for 24-72 hours before use to promote blast formation and replication of T-cells.

II. SPECIMEN REQUIREMENTS

Whole blood anticoagulated with heparin may be used (heparin should be at a concentration of 600 units/mL). The volume drawn is 120-240 mL.

Leukocyte concentrates (buffy coats) can be obtained from American Red Cross and is usually anticoagulated with EDTA or CPD. This is a unit of whole blood from which most of the plasma and red blood cells have been removed. The usual volume is 30 -50 mL.

For use of either of these sources of blood, the blood should be stored at room temperature and processed within 30 hours of collection.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.33 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate, or manufacturer's outdate, whichever comes first.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Observe manufacturer's outdate (one month after opening)

Sterile PHA-P - available from DIFCO desiccated in 5 mL vials (50 mg).

a. Add 5.0 mL of sterile water to vial. Mix until contents are dissolved. Add 45 mL HBSS to contents of vial. Mix well. Final concentration = 1000 µg/mL (200X concentration).

b. Divide into 1.0 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Label with date prepared and store at -20^0C. Observe manufacturer's outdate. Thaw as needed. Use 0.5 mL per 100 mL of media = 5µg/mL

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

Human IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL at -20^0C).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium = 100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see V. Procedure below.

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes
Sterile 50 mL conical tubes
Sterile 2, 5, 10, and 25 mL pipettes
Hemacytometer
25 and 75 cm^2 tissue culture flask
Sterile 500 mL bottles
Sterile 1.5 and 0.5 mL microcentrifuge tubes
20 µL, 200 µL and 1000 µL micropipettors
Sterile 200 µL and 1000 µL pipette tips
Bleach (household bleach diluted 1/100 with tap water)
Laminar flow hood (Class 2 biosafety hood)
Centrifuge capable of speeds up to 800 x g and equipped with a horizontal rotor and O- ring sealed safety cups
Compound microscope
CO_2 incubator (37 ± 1^0C with humidity)
37^0C and 56^0C water baths
Pipette aid
Sterile 60 mL syringes
Sample Site Coupler

V. PROCEDURE

1. Twice a week the lab receives blood for donor preparation. If a leukocyte concentrate prepared from a whole blood unit from the Red Cross is received, it will be tested by the Red Cross for anti-HIV, as well as hepatitis B and syphilis. Testing may not be complete when the unit is released, in which case, the Red Cross will call those results as soon as they are available. If heparinized whole blood is received it may be treated in the same way as the leukocyte concentrate, but the volume will be higher and the number of necessary tubes for white cell separation will be greater.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

2. Remove the cells from the bag, using a sample site coupler and 60 mL syringe.

3. Separate PBMC from the blood as follows:

   a. Accuspin Method: Carefully pour 20-30 mL of blood into large Accuspin tubes (as many as needed). Centrifuge the tubes at room temperature at 800 g for 20 minutes.

   b. Overlay Method: Add one part PBS or HBSS to one part blood. Blood should be carefully overlaid at a ratio of 4 parts diluted blood to 3 parts Ficoll reagent in 50 mL sterile tubes, being careful not to disturb the interface. Centrifuge tubes at room temperature at 400 x g for 30 minutes.

4. After centrifugation, remove cloudy interface (PBMC layer) into appropriately labeled 50 mL centrifuge tubes.

5. Wash cells by filling tubes with sterile PBS or HBSS and centrifuge at 400 x g for 10 minutes.

6. Decant supernatant after centrifugation, resuspend cells and fill tubes with sterile PBS or HBSS and wash again.

7. Resuspend each pellet in 10-30 mL of Growth Medium, depending on whether whole blood or leukocyte concentrate was used.

8. Count and record the number of viable PBMC/mL.

a. Pipette 10 µL of the sample into a 0.5 mL microcentrifuge tube, add 90 µL of trypan blue stain and mix.

b. Load a hemacytometer and count the number of PBMC in the four large cells.

c. Calculate the number of PBMC/mL: $\frac{\text{PBMC in all four squares}}{4} \times 10^5$

Example: $\frac{88}{4} \times 10^5 = 2.2 \times 10^6$ PBMC/mL

9. Place the cells in 75 cm^2 flasks (number of flasks depending upon workload for the week) at a concentration of 2 x 10^6/mL in Growth Medium. Total volume in each flask may be from 40-120 mL.

10. Add PHA-P at a final concentration of 5 µg/mL (e.g., 200 µL/ 40 mL medium).

11. Incubate at 37^0C, 5% CO_2 with humidity for 1-3 days before use..

VI. QUALITY CONTROL

Set up a qualitative HIV culture using the newly prepared donor PBMC as "patient cells" to verify that the new donor is HIV culture negative. (See - Qualitative PBMC Macrococulture Method.)

Do not use PHA-stimulated donor PBMC older than 3 days post stimulation.

VII. REFERENCES

Levy JA, Shimabukuro J. Recovery of AIDS-associated retroviruses from patients with AIDS or AIDS-related conditions and from clinically healthy individuals. J Infect Dis 1985;152:734-8.

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